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High-affinity RNA-binding domains of alfalfa mosaic virus coat protein are not required for coat protein-mediated resistance

(cross-protection/engineered resistance)

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ABSTRACT **A virus-based vector was used for the transient expression of the alfalfa mosaic virus coat protein (CP) gene in protoplasts and plants. The accumulation of wild-type CP conferred strong protection against subsequent alfalfa mosaic virus infection, enabling the efficacy of CP mutants to be determined without developing transgenic plants. Expression of the CP mRNA alone without CP accumulation conferred weaker protection against infection. The activity of the N-terminal mutant CPs in protection did not correlate with their activities in genome activation. The activity of a C-terminal mutant suggested that encapsidation did not have a role in protection. Our results indicate that interaction of the CP with alfalfa mosaic virus RNA is not important in protection, thereby leaving open the possibility that interactions with host factors lead to protection.**

In the past decade, many approaches have been investigated to create plants resistant to pathogens. Virus resistance has been conferred on plants by the expression of viral capsid or coat protein (CP) genes, replicase and movement protein genes, and viral antisense RNA (1). Of these approaches, CP-mediated resistance has been most widely investigated and is near commercialization (1–3). Alfalfa mosaic virus (AMV) CP and the CPs of many other plant viruses have been incorporated into the genomes of plants to provide protection (1, 4). This approach is based on the phenomenon of cross-protection, whereby a plant infected with a mild strain of virus is protected against a more severe strain of the same virus (5). The phenotype of the resistant transgenic plants includes fewer centers of initial virus infection, a delay in symptom development, and low virus accumulation. Protoplasts from virus-resistant transgenic plants are also resistant, suggesting that the protection is largely operational at the cellular level. Transgenic plants expressing AMV or tobacco mosaic virus (TMV) CP are protected against infection by virus particles but are susceptible to viral RNA, indicating that the protection may primarily involve an inhibition of virus uncoating (2).

AMV is a positive-strand RNA virus with coding capacity for four proteins. Virions contain three genomic RNAs and a subgenomic RNA (RNA4), which codes for the CP. The 24-kDa CP is the smallest of the virus proteins and has roles during early, intermediate, and late stages of infection. The N terminus of the CP is necessary for genome activation, in which the CP binds to the 3′ ends of the virus RNAs to provide stability vital for infection (6, 7). The CP may also function as part of the replication complex (8). The C terminus of AMV CP is essential for virion assembly (9).

We are investigating the activities of various AMV CP mutants in order to understand the mechanism of CP-mediated resistance. CP–RNA interactions have been suggested to be important in the resistance (2, 3). Therefore, we introduced mutations into regions of AMV CP that have been identified to be important for AMV CP–RNA interactions. The mutants were transiently expressed in protoplasts using a TMV-based vector so that we could rapidly evaluate the efficacy of mutant CPs without the need to develop and select transgenic lines of plants.

MATERIALS AND METHODS

DNA Constructions. pSP65A4 (6), containing a full-length cDNA of AMV RNA4, was used for the construction of mutant RNA4 cDNAs with changes in the CP coding region (Fig. 1*A*). The plasmid pCPΔATG was made by PCR using a first-strand primer described previously (6) and 5′-CCCTG-AATTCGTTTTTATTTTTAATTTTCTTTCAATTAC-TTCCATCTCGAGTTCTTC-3′ as second-strand primer to abolish the start codon and introduce an *Xho* I site (underlined). The *Eco*RI–*Bam*HI fragment of RNA4 in pSP65A4 was replaced by the mutant *Eco*RI–*Bam*HI fragment. The plasmid pCPΔATG(−) was made from pCPΔATG by inverting the *Bam*HI–*Sma* I fragment. The *Eco*RI–*Pst* I fragment, containing the inverted region, was cloned between the *Eco*RI and *Bst*XI sites in pSP65A4 to provide a downstream *Sma* I site. Plasmids pCPΔN2 and pCPΔN17 were made from pCPΔATG by using the first-strand primer (6) and 5′-CCATCTCGAGT-TCT**ATG**TCACAAAAG-3′ or 5′-CCATCTCGAGTTCTT-CACAAAAGAAAGCTGGTGGGAAAGCTGG-TAAACCTACTATACGT**ATG**TCTCAGAAC-3′ primer, respectively, which contain *Xho* I sites (underlined) and repositioned ATG codons (bold type). The *Xho* I–*Bam*HI fragment in pCPΔATG was replaced by mutant *Xho* I–*Bam*HI fragments. Thus, the CP genes in pCPΔN2 and pCPΔN17 lack codons for aa 3–4 and 3–19, respectively. Plasmids pCPN2A and pCPN2G were made similarly from pSP65A4 by using 5′-TACTCTCGAGATGGCTTCTTCACA-3′ or 5′-TACTC-TCGAGATGGGTTCTTCACA-3′ as the second-strand primer, respectively, to change the codon at the second amino acid position in CP to that of Ala or Gly. Plasmids pCPΔC19 and pCPΔC134 were made by introducing frameshifts in pSP65A4 at the *Apa* I or *Bam*HI site, respectively. All changes in the cDNAs were confirmed by restriction mapping or by sequencing. To introduce the AMV CP gene into the TB2 vector (Fig. 1*B*), the *Eco*RI–*Rsa* I fragment from RNA4 mutants or the *Eco*RI–*Sma* I fragment from pCPΔATG(−) was subcloned into the unique *Xho* I site of TB2 (10).

Protection Assay in Protoplasts and Plants. AMV strain 425 virions and RNA were purified (4). Capped vector transcripts were synthesized from the TB2 cDNA constructs (6, 10). Protoplasts (*Nicotiana tabacum* cv. Xanthi-nc) were isolated, inoculated with the vector transcripts (11), and assayed by an immunofluorescence assay using antiserum to TMV CP to determine the percentage of vector-infected protoplasts (6). This antiserum recognizes o-CP encoded by the vector (Fig. 1).

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Abbreviations: AMV, alfalfa mosaic virus; TMV, tobacco mosaic virus; CP, coat protein; WT, wild type.

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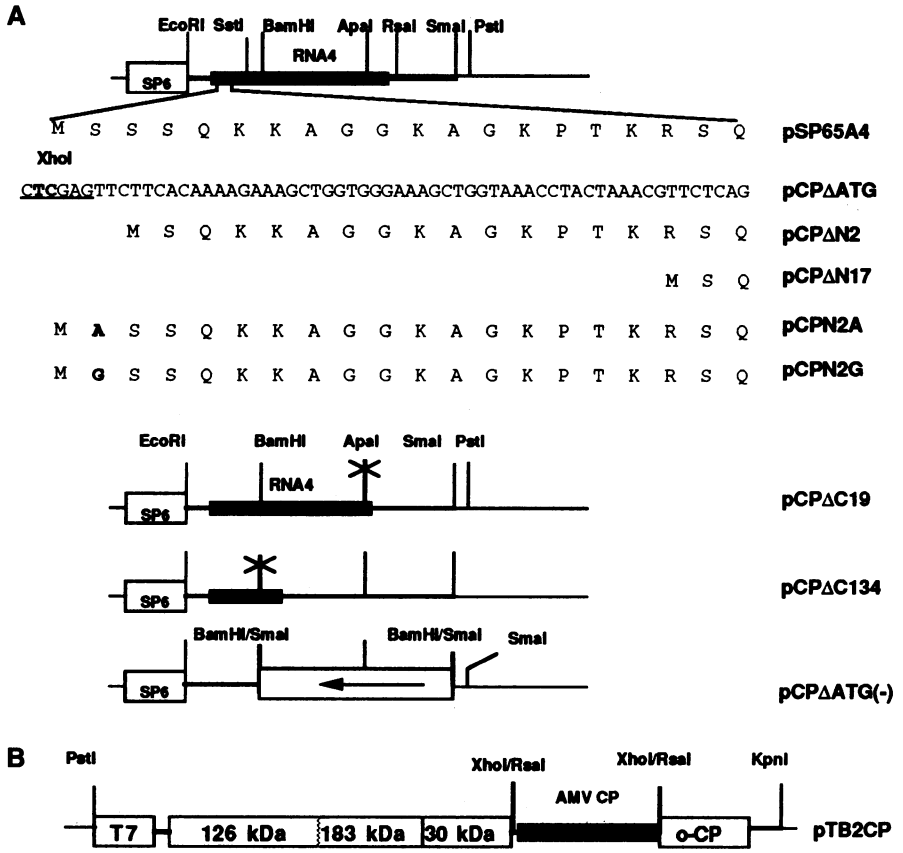

FIG. 1. (*A*) Mutations in the CP gene of AMV. The deduced amino acid sequence of the CP gene is indicated below the diagram of plasmid pSP65A4. The CP open reading frame is indicated by the black box; the bacteriophage SP6 promoter is indicated by the open box. The 5′ nucleotide sequence of the CP gene in pCPΔATG is indicated with the engineered *Xho* I site underlined; amino acid sequences of CP mutants with changes in the N-terminal region are indicated with amino acid substitutions in bold type. Frameshifts and insertions in the CP gene for other mutants are indicated on diagrams. A slash (/) between two restriction sites indicates that the sites were joined by blunt-end ligation without a change in the open reading frame, except in pCPΔAGT(−); † indicates that a frameshift was created at the restriction site. (*B*) Diagram of the TMV-based vector containing an AMV CP gene. The T7 bacteriophage promoter, TMV genes, and the sequence encoding odontoglossum ringspot virus CP (o-CP) are indicated by open boxes; the AMV CP open reading frame is indicated by the black box.

To determine the susceptibility of vector-infected protoplasts to AMV or AMV RNA, the protoplasts were inoculated 4 hr after transfection with AMV particles or RNA. Twenty-four hours after inoculation, AMV infection was assayed by Northern blot analysis or by the immunofluorescence assay using antiserum to AMV CP (4, 6). Statistical analysis of data was performed with SUPERANOVA (Abacus Concepts, Berkeley, CA). To test protection in plants, young leaves of *Nicotiana* sp. were infected with vector transcripts at ≈20 μg per leaf and inoculated 4 days later with AMV. AMV RNA accumulation was determined in inoculated and young uninoculated leaves. ELISA was used to determine the accumulation of CP in plants following transfection with vector RNA transcripts (4).

AMV RNA and CP Analysis. AMV CP accumulation was detected by Western blot analysis using monoclonal antibodies to AMV CP (4). AMV RNA accumulation was detected by Northern blot hybridization (4) using AMV cDNA probes to detect AMV genomic RNAs exclusive of the RNA4 sequence coding for CP. The relative density of bands on autoradiographs of Northern blots or on Western blots was determined by scanning digitized images with IPGEL software (Signal Analytics, Vienna, VA) to determine relative accumulation of AMV RNA or CP.

RESULTS AND DISCUSSION

Expression of AMV CP Mutants. RNA4 mutants transcribed from the mutant cDNAs (Fig. 1) by SP6 polymerase were tested for messenger activity in an *in vitro* wheat germ translation system. All mutant RNA4s, except for CPΔATG(−), which contains an inverted gene, CPΔATG and CPΔC134, directed the synthesis of proteins of the expected size (data not shown). The mutant CPΔATG lacks the native start codon of CP; thus, our results suggest that the downstream AUG at codon 95 is not functional. The failure to detect the synthesis of mutant CPΔC134 protein is consistent with previous results (6). All RNA4 mutants were subcloned into the TB2 vector (Fig. 1*B*). The TMV and the o-CP gene products of TB2 are required for TB2 replication, cell-to-cell movement, and encapsidation. The AMV CP gene is positioned behind the TMV CP subgenomic promoter so that there will be strong expression of a subgenomic mRNA coding for AMV CP. Therefore, AMV CP synthesis should occur 2–4 hr after inoculation, as in TMV-infected tobacco protoplasts (12). The 3′ untranslated region of AMV RNA4, containing strong CP binding sites, was not included in the constructs, except in pTB2CPΔATG(−). Thus, AMV CP should neither specifically bind to nor encapsidate TB2 RNA *in vivo*. Transfection of protoplasts with transcripts from the expression vectors resulted in infection of 70–80% of the protoplasts as indicated by the immunoassay. Fig. 2 shows that all mutant AMV CPs accumulated *in vivo* upon infection of protoplasts, except for CPΔC134, which is consistent with the *in vitro* translation results. Densitometer analysis of the blot in Fig. 2, adjusted for the number of infected protoplasts per lane, indicated that the accumulation of wild-type (WT) and mutant CPs in protoplasts infected with transcripts was 30–58% of that in AMV-infected protoplasts.

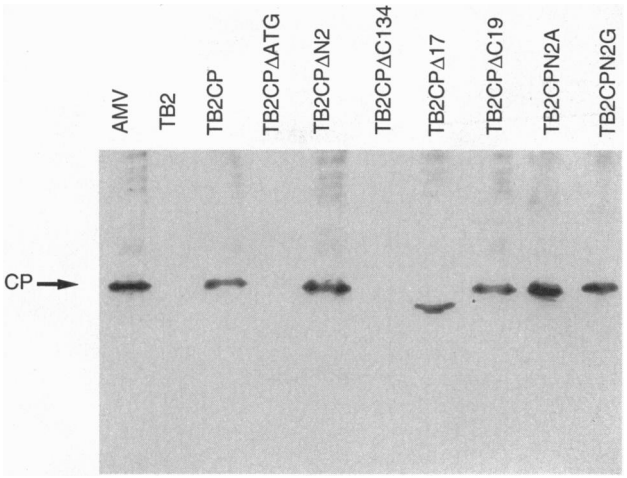

FIG. 2. CP accumulation in tobacco protoplasts infected with vector transcripts containing CP genes. Each lane of the Western blot (4) contained total protein from 2500 (first five lanes from the left), 3000 (next three lanes) or 5000 (last two lanes) infected protoplasts, collected 24 hr after inoculation. The specific vector transcript used for transfection of each protoplast sample is indicated above the lane; AMV indicates that the protoplasts were inoculated with virions.

These results indicate that the vector transcripts can be used to express WT and mutant AMV CPs in protoplasts.

Susceptibility of Protoplasts Accumulating AMV RNA or CP. Transfection by the vector transcripts followed by AMV inoculation was required to determine the efficacy of the CP mutants. Fig. 3*A* shows that an initial inoculation with TB2 transcripts (bar 2) or mock inoculation (bar 1) followed by AMV inoculation resulted in AMV infection in 80–89% of the protoplasts. Thus, the accumulation of TMV and o-CP gene products did not protect the protoplasts from AMV infection. However, inoculation with TB2CP transcripts conferred significant protection against AMV so that only 25% of the protoplasts became infected (Fig. 3*A*, bar 3). Thus, expression of AMV RNA and/or CP conferred resistance. To determine whether RNA expression alone conferred protection, protoplasts were inoculated with transcripts containing untranslatable CP genes (Fig. 3*A*, bars 4–6). Expression of antisense RNA and sense RNA, without CP accumulation, conferred some protection so that 20–22% of the protoplasts did not become infected with AMV compared with the TB2-infected control. Only one of the untranslatable AMV CP genes contained the RNA 3′ untranslated region; therefore, it was not required for the protection. Others have reported that the expression of virus RNA sequences alone will confer resistance (13, 14). It has been suggested that a cellular mechanism that specifically degrades the virus RNA is induced in plants protected by RNA (15). It is possible that a similar mechanism accounts for the protection conferred by the expression of AMV CP mRNA in our protoplasts. However, our protoplast system differs significantly from the transgenic plants used in previous studies. AMV RNA expression in the protoplasts occurred only in the cytoplasm, without direct nuclear involvement. Transgene *in planta* expression, however, is controlled by nuclear transcription, processing, and export and occurs throughout the life of the plant. Hence, additional studies are needed to determine whether similar mechanisms are involved.

The percentage of infected protoplasts reflects the number of protoplasts in which sufficient quantities of the CP have accumulated for detection by AMV CP-specific antibodies. The accumulation of CP in TB2CP-infected protoplasts resulted in a low-intensity fluorescence in the immunoassay, whereas protoplasts infected by AMV fluoresced brightly. To confirm that the percentage of AMV-infected protoplasts determined by the immunoassay correlated with the accumulation of virus, AMV RNA levels were determined by Northern blot analysis. Fig. 3*B* shows that AMV RNA accumulation in mock-inoculated or in TB2-infected protoplasts was much greater than that in TB2CP or TB2CPΔATG-infected protoplasts. This agrees with the results shown in Fig. 3*A*. Densitometer analysis of the blot indicated that the amount of AMV RNA in TB2CP and TB2CPΔATG-inoculated protoplasts was 35% and 49% of that in the TB2 control protoplasts, respectively. These levels of RNA are consistent with the results of the immunoassay of this experiment, which indicated that the percentage of AMV infection in the TB2CP and TB2ΔATG-inoculated samples was 28% and 54% of that in the TB2 control. This suggests that AMV accumulation occurred only in those protoplasts detected by the immunoassay. Therefore,

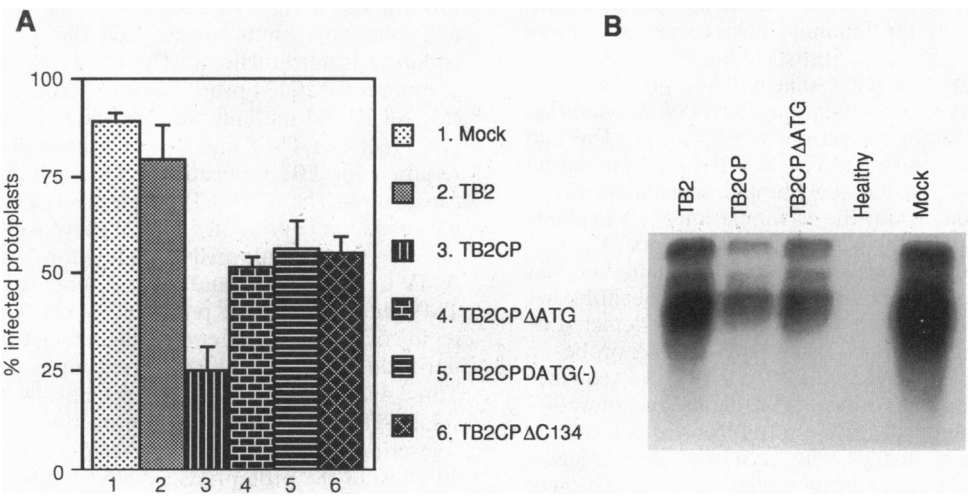

FIG. 3. Susceptibility of tobacco protoplasts after transfection with vector transcripts. Protoplasts were first inoculated with transcripts from various vector constructs at 5 μg per 10^5 protoplasts and then inoculated 4 hr later with AMV at 3 μg per 10^5 protoplasts. (*A*) Percentage of protoplasts infected with AMV at 24 hr after AMV inoculation. The key at right indicates the specific vector transcript used for the transfection. Control protoplasts (Mock) received no added transcript during transfection. Bars indicate mean and SD from at least four experiments. (*B*) AMV RNA accumulation in vector-infected protoplasts subsequently inoculated with AMV. AMV RNAs were detected by Northern blot hybridization with a probe consisting of ^{32}P-labeled AMV cDNAs that did not contain CP sequences. Each lane contained 5 μg of protoplast RNA isolated 24 hr after AMV inoculation. The specific vector transcript used for transfection of each protoplast sample is indicated above the lane.

the immunoassay was used to analyze all protoplast experiments.

The use of the protoplast system for analysis of protection is rapid and reproducible. The reduction in replication reflects resistance at the level of individual cells which should be predictive of resistance in whole plants. To determine whether this assumption is true, the susceptibility of TB2CP-infected *Nicotiana benthamiana* and *N. tabacum* cv. Xanthi-nc plants to AMV infection was investigated. AMV, TB2, and TB2CP systemically infect *N. benthamiana*. However, only AMV routinely systemically infects Xanthi-nc, whereas TB2 and TB2CP replicate primarily in inoculated leaves with limited systemic spread. Inoculation of TB2 or TB2CP transcripts into tobacco plants was followed by AMV inoculation 4 days later. Six days after AMV inoculation, similar amounts of AMV RNA were in plants initially mock-inoculated or inoculated with TB2 (Fig. 4). Thus, without AMV CP gene expression, no protection was conferred. In contrast, TB2CP-infected plants accumulated only small amounts of AMV RNA. Moreover, AMV could not be detected in the young uninoculated leaves of these plants as it was in the TB2-infected plants. The accumulation of AMV RNAs in the protected plants inoculated with the higher concentration of AMV (Fig. 4*B*) was greater than that in plants inoculated with the lower concentration (Fig. 4*A*), in agreement with earlier reports that CP-mediated protection is less effective at higher inoculum concentrations (2).

These results indicate that the resistance conferred by TB2CP infection is stronger in plants than in protoplasts. This greater resistance is probably due to greater accumulation of CP in plants than in protoplasts. ELISA indicated that TB2CP-infected *N. benthamiana* plants contained AMV CP at 200–800 ng/mg of protein in the inoculated leaves by 7 days after transfection. This is similar to the accumulation of CP in virus-resistant transgenic plants (4). These results indicate that the resistance detected by the protoplast assay is applicable to whole plants; therefore, further studies were carried out with protoplasts.

Effect of Changes in CP on Protection. C-terminal changes. A deletion of 134 aa in CPΔC134 affected the expression and/or accumulation of the truncated protein, since the protein could not be detected *in vitro* or *in vivo* (Fig. 2). The protection conferred by this mutant was similar to that conferred by the expression of RNA alone (Fig. 3*A*). A mutant with a C-terminal deletion of 19 aa, CPΔC19, accumulated in protoplasts (Fig. 2). This mutant (Fig. 5, bar 6) conferred protection similar to that of WT CP (bar 3). Therefore, the C-terminal 19 aa are dispensable for protection. AMV containing a similar mutant CP gene did not effectively produce virions during infection (9). Therefore, taken together, the data suggest that AMV CP need not contain regions essential for the assembly of virions to be active in mediating protection. A similar result was recently reported for an assembly-defective mutant CP of TMV, which conferred protection (24).

N-terminal changes. The N-terminal deletion mutants CPΔN2 and CPΔN17 (Fig. 5, bars 4 and 5) behaved similarly to WT CP (bar 3) in conferring protection, indicating that the N-terminal 17 aa of the CP are dispensable for protection. A report indicating that a Ser-to-Gly change at the second amino acid in AMV CP abolished the activity of the CP in protection in transgenic plants (16) led us to test two additional mutants. A Ser-to-Ala change at the second position in the CP did not affect the level of protection (Fig. 5, bar 7). An identical substitution in the CP of two potyviruses, likewise, had no effect on their efficacy in protection in transgenic plants (17, 18). However, a change to Gly greatly reduced the protection due to AMV CP (Fig. 5, bar 8), in agreement with the previous report (16). The protection conferred by CPN2G was not statistically different from that conferred by the expression of CP mRNA alone (Fig. 3*A*). It was recently reported that transgenic plants expressing AMV CP with the Ser-to-Gly change were resistant to an AMV mutant with an identical change, but not to WT AMV (19). It was suggested that the difference in activities of the WT and mutant CPs was due to different affinities for a host component. Perhaps this is due to differences in N-acylation. Ser and Ala are similarly acylated, whereas Gly, which occurs less commonly at the second amino acid position, is specifically myristoylated (20, 21). Perhaps the specific moiety attached to the CP by acylation is critical for interaction with host components and, consequently, for the development of resistance.

Effect of CP Mutations on the Interaction Between CP and AMV RNA. The N-terminal 25 aa of CP are necessary and sufficient for genomic activation (22). To determine whether the activity of CP mutants in protection correlates with their activity in genome activation, protoplasts were inoculated with RNA4 transcripts of mutant CP genes together with genomic RNAs. Table 1 shows that genome activation was dependent upon the presence of the N terminus in CP. From these results and those shown in Fig. 5, we conclude there is no correlation between the activity of CP mutants in protection and their ability to activate the genome.

Susceptibility of Protoplasts Accumulating CP to AMV RNA Infection. To determine whether the TB2CP-infected protoplasts were resistant to infection by RNA as well as by virions, protoplasts were challenged with a mixture of all four AMV RNAs. Inoculation of protoplasts infected with TB2CPΔN17 or TB2CPΔC19, which are resistant to AMV, or mock-inoculated control protoplasts, which are susceptible to AMV, resulted in infection of 30–40% of the protoplasts as determined by the immunoassay. Thus, the protected proto-

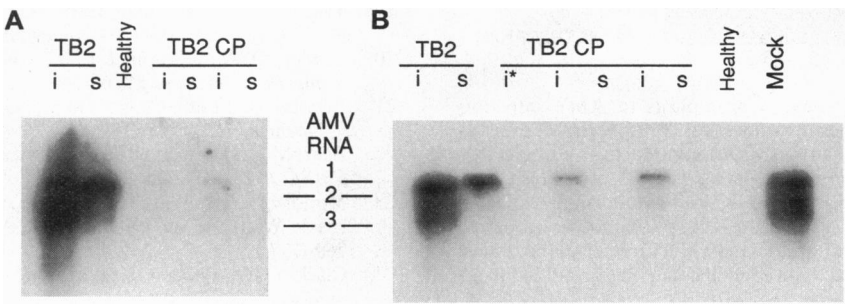

FIG. 4. Susceptibility of tobacco plants following transfection with vector transcripts. Leaves of *N. benthamiana* (*A*) or *N. tabacum* cv. Xanthi-nc (*B*) were inoculated with TB2 or TB2CP transcripts followed 4 days later by AMV at 6 or 18 μg/ml, respectively. AMV RNA was detected by Northern blot hybridization as in Fig. 3. Each lane contained 7 μg of total RNA isolated 6 days after the AMV inoculation. The lanes are labeled with the specific transcript used for the transfection. Control plants (Mock) were inoculated with buffer. An asterisk indicates that the sample was from a TB2CP-infected plant that was not challenged by inoculation with AMV; "i" indicates that the sample was from inoculated leaves; "s" indicates that the sample was from young uninoculated leaves; "Healthy" indicates that the sample was from an uninfected plant. Duplicate "i" and "s" samples came from different plants.

Table 1. Activity of AMV CP mutants in genome activation and protection

CP	Relative genome activation,* %	Activity in CP-mediated protection†
WT	100	+
CPN2A	69	+
CPN2G	73	−
CPΔATG	0	−
CPΔN2	92	+
CPΔN17	0	+
CPΔC19	14	+

*Inoculum consisted of 0.5 μg of genomic RNAs plus 3 μg of WT or mutant RNA4 per 10^5 protoplasts. Activity in genome activation is expressed relative to WT CP (100%).
†From the data in Fig. 5; +, protection similar to that conferred by WT CP; −, protection similar to that conferred by CP mRNA alone.

plasts were as susceptible to RNA as were the controls, in agreement with previous results with transgenic plants in our laboratory (4). However, these results are not in agreement with reports that transgenic plants expressing another strain of AMV CP were resistant to both AMV virions and RNA (16, 19). The reasons for this difference are unknown.

In conclusion, our data indicate that expression of the AMV CP gene from the TMV vector inhibits AMV infection. Both RNA and protein are involved in this inhibition. RNA may confer protection by induction of a cytoplasmic pathway for degradation of AMV RNA; however, we cannot exclude the possibility that AMV RNA translation and replication are inhibited by RNA duplex formation. The accumulation of full-length WT AMV CP and a deletion "core" protein confer stronger protection than does the accumulation of AMV RNA alone. The N and C termini of AMV CP, which contain regions that are important for well-documented CP–RNA interactions, can be removed without effect on protection. Results showing that there is specificity at the second amino acid position suggest that the type of acylation may affect the activity of the protein in protection. Thus, our results indicate that protection does not involve CP–RNA interactions and leave open the possibility that interactions with host components mediate protection. Moreover, protection was circumvented by RNA inoculum in the TB2CP-infected protoplasts, as in our transgenic plants, which indicates that virus uncoating was inhibited. Thus, ribosomal proteins involved in uncoating during cotranslational disassembly (23) are likely host components for the interaction with AMV CP leading to protection.

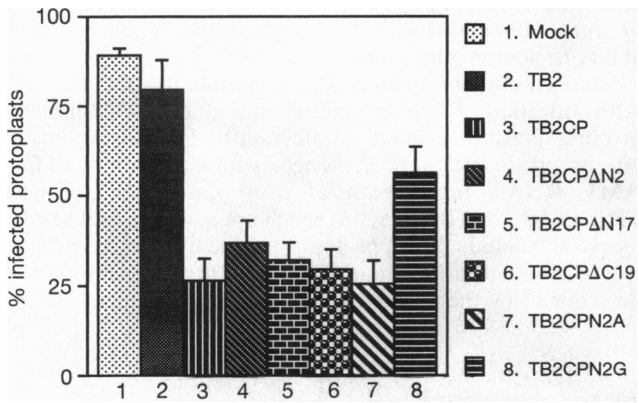

FIG. 5. Susceptibility of tobacco protoplasts to AMV infection following transfection with vector transcripts. Protoplasts were inoculated with transcripts from various vector constructs at 5 μg per 10^5 protoplasts followed 4 hr later by AMV at 3 μg per 10^5 protoplasts. The percentage of protoplasts infected with AMV was determined at 24 hr after AMV inoculation. The key indicates the specific vector transcript used for transfection. Control protoplasts (Mock) received no added transcript RNA during transfection. Bars indicate mean and SD from at least five experiments.

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